

REMARKS

Claims 12-17 were pending prior to this Response. By the present communication, no claims have been added, canceled, or amended. Support for the amended claims may be found in the specification as filed. As such, the amendments do not raise any issues of new matter and the amended claims do not present new issues requiring further consideration or search. Accordingly, upon entry of the present amendment, claims 12-17 will be pending in this application.

Rejections under 35 U.S.C. §112, First Paragraph

Applicants respectfully traverse the rejection of claims 12-17 under 35 U.S.C. §112, first paragraph, as allegedly failing to comply with the enablement requirement. Specifically, the Office Action alleges that at the time the application was filed, the art of administering transduced neural progenitor cells to an individual so as to provide a tangible therapeutic benefit was poorly developed and unpredictable.

Attached herewith as Exhibit A is an article describing intracerebral administration of nerve growth factor (NGF) by transplants of NGF-secreting immortalized neural progenitor cells in the nucleus basalis and septum of behaviorally impaired aged rats (Martinez-Serrano, et al., (1998) *Proc. Natl. Acad. Sci. USA*, 95, 1858-1863). Exhibit A indicates that, “[w]ork during the last few years has shown that *ex vivo* gene transfer techniques can provide an interesting alternative for long-term delivery of therapeutically active proteins...” (Exhibit A, page 1858, col. 2, citing Martinez-Serrano, et al., (1995) *Neuron* 15, 473-484; and Martinez-Serrano, et al., (1996) *Proc. Natl. Acad. Sci. USA* 93, 6355-6360, which is attached as Exhibit B). Thus, the results shown in Exhibit A “demonstrate the usefulness and success of the *ex vivo* gene transfer approach for long-term intracerebral delivery of neurotrophic factors, as compared with other alternative methods of administration.” (Exhibit A, page 1862, col. 1).

Attached herewith as Exhibit C is an article describing the use of immortalized suprachiasmatic nucleus (SCN) cells to generate robust rhythms in uptake of the metabolic marker 2-deoxyglucose and in the content of neurotrophins. (Earnest, et al., (1999) *Science* 283,

693-695). Exhibit C indicates that, “[l]ike other cell lines and many peripheral insect and mammalian tissues, [the] immortalized cells derived from the SCN generate circadian rhythms *in vitro*. (Exhibit C, page 694, col. 3).

Finally, the Office Action alleges that Mehler, et al., state that it may be necessary to promote lineage commitment of progenitor cells *in vitro* prior to transplantation. (Office Action, page 4). Applicants submit that the same reference also discloses that, “[t]ransplantation of wild-type or genetically engineered immortalized progenitor cells has also been used experimentally to improve biochemical and morphologic parameters in mice with specific lysosomal storage diseases....” (Mehler, page 783, col. 1). Mehler further discloses that, “[i]n addition, within the spinal cords of myelin-deficient rats, these immortalized progenitor cells can proliferate, migrate, and myelinate axons for several weeks following transplantation.” (Mehler, page 783, col. 1).

MPEP §2164.05(b) states that “[t]he relative skill of those in the art refers to the skill of those in the art in relation to the subject matter to which the claimed invention pertains at the time the application was filed... *In re Naquin*, 398 F.2d 863, 866, 158 USPQ 317, 319 (CCPA 1968)”. Applicants submit that the specification as filed describes that “[c]ells may be introduced by, for example, stereotaxic implantation or intracerebral grafting into the CNS of patients. The cells themselves may have the capacity to functionally replace neurons that die in neurodegenerative disorders, or may serve as sources of agents (such as trophic factors) that have therapeutic benefit.” (Specification, page 19, lines 24-27). Thus, the skilled artisan at the time of the filing of the present application and in view of the above-referenced Exhibits, would have understood that *ex vivo* gene transfer has been successfully demonstrated in accepted mammalian animal models. The state of the art existing at the filing date of the application is used to determine whether a particular disclosure is enabling as of the filing date. *Chiron Corp. v. Genentech Inc.*, 363 F.3d 1247, 1254, 70 USPQ2d 1321, 1325-26 (Fed. Cir. 2004) (“a patent document cannot enable technology that arises after the date of application”). See MPEP §2164.05(a). Accordingly, Applicants submit that at the time of filing, transplantation of immortalized neural progenitor cells was known and understood. Thus, the claimed invention is enabling as of the filing date of the application.


CONCLUSION

In summary, for the reasons set forth herein, Applicants submit that the pending claims clearly and patentably define the invention and respectfully request that the Examiner withdraw all rejections and pass the application to allowance. If the Examiner would like to discuss any of the issues raised in the Office Action, the Examiner is encouraged to call the undersigned so that a prompt disposition of this application can be achieved.

No fee is believed to be due in connection with the filing of this paper. However, the Commissioner is hereby authorized to charge any fees that may be required by this paper, or credit any overpayment to Deposit Account 07-1896 referencing the above-identified attorney docket number.

Respectfully submitted,

Date: December 19, 2007



Antony M. Novom, J.D.
Reg. No. 45,517
Telephone: (858) 638-6641
Facsimile: (858) 677-1465

DLA PIPER US LLP
4365 Executive Drive, Suite 1100
San Diego, California 92121-2133
USPTO CUSTOMER NO. 28213

Ex vivo nerve growth factor gene transfer to the basal forebrain in presymptomatic middle-aged rats prevents the development of cholinergic neuron atrophy and cognitive impairment during aging

(gene therapy/Alzheimer's disease/memory/p75^{NTR})

ALBERTO MARTÍNEZ-SERRANO*^{†‡} AND ANDERS BJÖRKLUND*

*Wallenberg Neuroscience Center, University of Lund, Sölvegatan 17, S-223 62-Lund, Sweden; and [†]Center of Molecular Biology "Severo Ochoa," Autonomous University of Madrid, Campus Cantoblanco, 28049-Madrid, Spain

Communicated by Per O. Anderson, University of Oslo, Oslo, Norway, December 1, 1997 (received for review July 17, 1997)

ABSTRACT Nerve growth factor (NGF) is able to restore spatial learning and reverse forebrain cholinergic neuron atrophy when administered intracerebrally to behaviorally impaired aged rats. In the present study, behaviorally unimpaired, middle-aged rats (14–16 months old) received transplants of *ex vivo* transduced, clonal NGF-secreting immortalized neural progenitor cells, bilaterally in the nucleus basalis and septum. During the subsequent 9 months the aged control animals developed the expected impairment in spatial learning in the water maze task, whereas the animals with NGF-secreting grafts maintained a performance level not different from the 12-month-old control rats. The marked age-induced atrophy (~25%) of the cholinergic neurons in medial septum and nucleus basalis, seen in the aged control rats, was not present in the NGF-treated aged animals. ³H-labeled thymidine autoradiography showed that the transduced cells survived well and had become integrated into the host tissue surrounding the injection sites, and reverse transcription-PCR analysis revealed expression of the NGF transgene, at both 4 and 9 months postgrafting, in the grafted tissue. The results show that long-term supply of NGF from *ex vivo* transduced immortalized neural progenitor cells locally within the nucleus basalis and septum can prevent the subsequent development of age-dependent neuronal atrophy and behavioral impairments when the animals reach advanced age.

Studies in rodents have shown that the basal forebrain cholinergic system undergoes progressive degenerative changes with advancing age, and that the magnitude of these changes generally is correlated with the severity of the behavioral impairments that the aged animals exhibit in various learning and memory tasks (1–3). Indeed, several lines of evidence indicate that normal cortical and hippocampal function depends on the modulatory afferent control exerted by the two principal basal forebrain cholinergic projection systems originating in the septal-diagonal band area and the nucleus basalis (1–4).

Injections or infusions of exogenous nerve growth factor (NGF) have been successfully used to reverse the age-dependent atrophic changes in the cholinergic forebrain neurons, as well as the performance of the aged animals in spatial memory tasks (5–10). NGF is a neurotrophic factor with potent neuroprotective effects on TrkA/p75^{NTR} receptor bearing cholinergic neurons in the central nervous system (CNS), both *in vitro* and *in vivo* (11–13). Although there is little evidence for a specific deficit of NGF in the brains of cognitively impaired animals (14, 15), the results obtained with acute administration of NGF in aged animals suggest that increased supply or availability of NGF to the

forebrain cholinergic neurons during aging may be able to stimulate their function and reverse their atrophic state.

The usefulness of intracerebroventricular or systemic administration of NGF may be limited by deleterious side effects such as hyperalgesia, hypophagia, and weight loss (16, 17). For this reason, long-term administration of NGF may require low-level, localized intraparenchymal delivery directly to the area containing the receptive neurons. Work during the last few years has shown that *ex vivo* gene transfer techniques can provide an interesting alternative for long-term delivery of therapeutically active proteins, which may circumvent the drawbacks associated with chronic intracerebral infusions. In aged rats, intracerebral transplantation of either fibroblasts (18) or immortalized neural progenitor cells (19, 20) engineered to secrete NGF has thus successfully been used to reverse cholinergic neuron atrophy and improve the aged rats' spatial learning performance in the Morris water maze task. These results show that cellular implants with an estimated secretion rate of 10–100 ng NGF/day, placed in either nucleus basalis or septum, are as efficient as intraventricular infusions of 1–6 µg NGF/day, indicating that local cell-based delivery is a highly efficient route of administration of neurotrophic factors. Polymer capsules containing NGF-secreting fibroblasts have yielded similar results (21), but in this case the capsules were implanted intraventricularly.

So far, all studies on intracerebral NGF delivery during aging have been concerned with amelioration of already established deficits in aged animals, and the maximum duration of intracerebral NGF delivery has been 4 weeks in the studies using injections or infusions (see table in ref. 21), and 10 weeks in the studies using *ex vivo* gene transfer (20). The present study was designed to investigate to what extent long-term local NGF delivery by transplants of NGF-secreting immortalized neural progenitor cells in nucleus basalis and septum, implanted in middle-aged rats at an early presymptomatic stage, can prevent the development of cholinergic neuron atrophy and behavioral impairments over the subsequent 9 months, when the animals have reached advanced age.

METHODS

Neural Stem Cells for *ex Vivo* Gene Transfer of NGF. The production and characterization of the control- or NGF-secreting clonal cell lines used in the present work have been described in detail elsewhere (22); the parental neural stem cell line was the E16 rat hippocampus-derived H19.5 cell line (23), made to express NGF by retroviral transduction, by using a construct without internal promoters or marker/selection genes. Cells were cul-

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

© 1998 by The National Academy of Sciences 0027-8424/98/951858-06\$05.00/0
PNAS is available online at <http://www.pnas.org>.

Abbreviations: RT, reverse transcription; NGF, nerve growth factor; CNS, central nervous system.

[‡]To whom reprint requests should be addressed at the present address: Center of Molecular Biology "Severo Ochoa," Laboratory CX-450, Autonomous University of Madrid, Cantoblanco, 28049-Madrid, Spain. e-mail: amserrano@trasto.cbm.uam.es.

tured in DMEM (GIBCO) supplemented with 10% fetal bovine serum, 2 mM glutamine, and 10,000 units/ml streptomycin/penicillin. Before grafting, the cells were labeled in culture for 72 h at $10 \mu\text{Ci/ml}$ with ^3H -labeled thymidine (Amersham); for grafting, a single cell suspension at $150,000 \text{ cells}/\mu\text{l}$ was prepared by trypsinization from near to confluent monolayers.

Animal Groups and Surgery. Eighty-three middle-aged (14- to 16-month-old) female Sprague-Dawley rats were tested in the Morris water maze for spatial navigation performance during 1 week, as previously described (19, 25), following a four-trials-per-day schedule (pregrat test in Fig. 1A). Forty-seven nonimpaired animals [defined as those showing a swim distance to find the hidden platform within the mean ± 2 standard deviations of the same score in a group of young (3-month-old) animals ($n = 10$)] were allocated to the following groups: intact ($n = 10$, no

transplantation surgery), control-graft ($n = 12$, receiving grafts of unmodified H1b5 cells), or NGF-graft ($n = 25$, receiving a transplant of the NGF-secreting clone of H1b5 named E8 in previous studies, ref. 22). Ten of the behaviorally impaired animals were used for expression studies (see below), and the rest were allocated to a different study.

All animals receiving grafts of neural stem cells were bilaterally implanted at both the medial septal (MS) and the nucleus basalis magnocellularis (NBM), receiving a total of 1.2 million cells (8 deposits $\times 150,000 \text{ cells per deposit}$). Details of the transplantation procedure can be found elsewhere (19, 20).

Behavioral Testing. The animals were studied for their performance in a working memory/learning task in the water maze test, as described below (19, 25). Two postgrafting rounds of testing in the water maze were undertaken at 4 and 9 months postsurgery, each consisting of 5 testing days with four trials per day. The hidden platform remained in the same quadrant of the pool for the duration of the experiment, and starting locations were randomized. Spatial probe trials (removed platform) conducted at the end of day 5 in each test were not informative enough in the present experiment because of the low training load (two to three crossings over the 10-cm diameter platform location).

Fourteen of the 47 animals died during testing, and at the time of perfusion noticeable spontaneously occurring pituitary tumors were found in 11 animals; they were, therefore, not considered in the final analysis. The final group composition was eight rats in the combined Intact+ control-graft groups (three intact and five control-grafts), and nine in the NGF-graft group. Intact and control animals were combined in a single group because they did not differ in either performance in any of the behavioral tests nor in the histological analysis. Ten adult animals (3 months old) were taken in parallel (12 months old at the end of the experiment).

Testing in the operant test apparatus for the standard delayed-matching-to-position (DMTP) task was performed as described (24); 6 intact, 6 control-graft, and 14 NGF-grafted animals randomly sampled from the whole groups were tested; learning of the task took 10 weeks for the aged animals (at an age of 20–23 months); no difference was observed between the groups either during the learning period or in the level of performance at the end of the test.

Histology. After completion of the experiment the animals were intracardially perfused with ice-cold buffered paraformaldehyde and their brains were sectioned for histological assessment of number and size of the forebrain cholinergic neurons after immunostaining for the p75^{NTR} neurotrophin receptor by using a monoclonal antibody generated from the 192-IgG hybridoma line (courtesy of E. Johnson, Washington University, St. Louis, MO). Sections were analyzed by using the CAST (computer-assisted stereological toolbox)-GRID software (Olympus, Denmark) operating an Olympus microscope equipped with a motorized stage. Both MS and NBM (defined as in refs. 19, 20, and 22) were bilaterally examined, and the results of the morphometric analyses are given as total cell counts for both sides combined.

Reverse Transcriptase (RT)-PCR Detection of Transgene Expression. Long-term *in vivo* expression of the NGF-coding retroviral vector and significant production of NGF bioactive protein by the grafted cells (assessed both as protein and bioactivity) have previously been demonstrated as long as 10 weeks postgrafting (19, 20, 22). To answer the question of whether or not the retroviral vector in the NGF-secreting neural stem cells could be expressed for the duration of the experiment at the target regions in the grafted brain, an independent group of 10 animals was bilaterally implanted into the NBM, as above, with control or NGF-secreting cells (left or the right hemisphere, respectively; septal grafts were not used because of the ease of contamination of the control-grafted hemisphere with NGF-cells at this location); one animal died during the 9-month postoperative

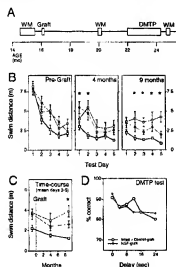


Fig. 1. Experimental design and behavioral testing. (A) Schematic illustration of the experimental schedule, from the pregrafting behavioral test in the Morris water maze (WM) to the last test, performed at 9 months postgrafting. During each WM test, individual animals were trained for 5 days, four trials per day; the boxes represent the actual time required to test all the animals participating in the experiment. (B) Performance in the WM tests (distance swum to find the hidden platform, mean \pm SEM), before grafting, and 4 and 9 months postgrafting. Open squares, adult rats; open circles, int+con-graft group; closed circles, NGF-grafted aged animals. For the 9-month postgrafting test, a two-way ANOVA reported significant differences among groups ($F_{2,27} = 11.69$, $P = 0.0003$). The asterisks in the diagram denote a significant difference among groups for individual testing days ($P < 0.05$, one-way ANOVA). For the 9-month test, int+con was different from adult at all days but the first one and different from the NGF-graft group at days 3 and 5; the NGF group was not different from adult animals at days 1 and 2, and 4 and 5. (C) Temporal changes in task performance (average of data from days 3–5) from mid- to advanced age. The int+con-graft group did not improve performance with consecutive tests, whereas both NGF-graft and adult groups did. A two-way ANOVA reported significant differences both among groups and between tests ($F_{2,27} = 14.96$, $P = 0.0001$, and $F_{2,27} = 3.65$, $P = 0.033$, respectively). One-way ANOVA indicated that all three groups were different from each other at the 9-month test ($P < 0.05$, posthoc Fisher PLSD). A one-tailed paired *t* test, comparing pregrat vs. 9-month test values yielded significant differences for NGF-graft and adult groups, but not for the int+con-graft group (int+con-graft, $P = 0.423$; NGF-graft, $P = 0.0159$; adult, $P = 0.0099$). The intact and control-graft groups did not differ in their performance in any of the tests ($P > 0.05$ in all cases). For C, the actual values for the 9-month test were: intact = 3.8 ± 1.03 m, control-graft = 3.95 ± 0.76 m. (D) Performance in the DMTP test in the operant test apparatus; experimental groups did not differ either in the performance with no delays (one-way ANOVA at 0 sec delay, $F_{1,16} = 0.09$, $P = 0.77$), nor at increasing delays (two-factor ANOVA: groups, $F_{1,16} = 0.0053$, $P = 0.94$; repeated measures, $F_{5,16} = 1.8619$, $P = 0.1121$). Overlapping SEM bars are omitted for clarity.

period, three rats were sacrificed at 4 months, and the remaining six were sacrificed at 9 months postgrafting. Dissected frozen tissue containing the NBM region was processed for RT-PCR of the retroviral mRNA (19, 20, 22); two micrograms of total RNA was reverse-transcribed to cDNA, and one-fourth of the RT products were subjected to amplification by PCR by using a primer set that specifically amplifies the retroviral transcript coding for mNGF in the NGF-stem cells (see ref. 22 for further methodological details and sequences). PCR products were separated by agarose electrophoresis, transferred to nylon membranes, and hybridized with a specific probe, POLYAS36, directed against the cloning site in the retroviral plasmid (22). Standards were generated by mixing known number of cells taken from fresh, dividing cultures with an equal weight of rat brain tissue (from intact animals) equivalent to the dissected pieces from grafted animals and run in parallel with the experimental samples.

RESULTS

NGF Prevents the Development of Cognitive Impairments with Aging. Middle-aged Sprague–Dawley rats were tested in the Morris water maze to select those showing no signs of impairments (within 2 SD of the young control group). Consistent with previous reports (25), we found 36 animals that were already impaired (43% of the population) and 47 non-impaired animals (57%). After grafting, the animals were subsequently tested for their performance in the same task 4 and 9 months later (see scheme in Fig. 1A), the last test being performed at the age of 23–25 months.

The control animals not receiving any extra neurotrophic support (int+con group) developed the expected deficits in the water maze task: by the end of the 9-month period, the distance swum to find the hidden platform was 4.26 ± 0.65 m, which was significantly longer when compared with the adult animals (0.84 ± 0.13 m) [$P < 0.05$, one-way ANOVA, post-hoc Fisher probable least-squares difference (PLSD)] (Fig. 1B). In contrast, the aged animals receiving NGF-producing cell implants maintained their performance at a level not significantly different from adult animals (swim distance = 1.97 ± 0.44 m, $P > 0.05$) and significantly different from the int+con-graft group ($P < 0.05$). Combined analysis of the three consecutive tests (average of data from days 3 to 5, Fig. 1C) indicated that both the adult and NGF-graft groups improved their performance as a consequence of increased training, resulting in a reduced swim distance (and escape latency) to find the hidden platform at the end of the experiment. In contrast, the int+con-graft rats did not improve their performance in spite of receiving the same training load (one-tailed, paired *t* test comparing the average performance of days 3–5 from the pregraft test to the 9-month test: adult, $P < 0.01$; aged int+con-graft, $P = 0.4231$; aged NGF-graft, $P = 0.0159$).

Swim speeds did not differ among experimental groups in any of the tests (day 5, int+con vs. NGF; pretest, $P = 0.1433$; 4-month test, $P = 0.6397$; 9-month test, $P = 0.3278$), which indicates that the differences in performance in the water maze task were not due to motor deficits, and that the improvements observed in the NGF-grafted rats are not due to NGF effects on this parameter. Motor coordination and attention were studied in more detail in an operant test apparatus by using the delayed-matching-to-position (DMPT) task, where both groups of aged rats reached more than 90% performance after a training period of 10 weeks (percentage of correct choices int+con-graft = 90.8 ± 5 , NGF-graft = 92.7 ± 3.7 , one-way ANOVA at 0 sec delay, $F_{1,16} = 0.09$, $P = 0.77$) (Fig. 1D); analysis of nose-poke frequency indicated the same degree of attention to the task in both groups of animals (1.59 ± 0.17 and 1.77 ± 0.15 pokes per sec, int+con-graft and NGF-graft group, respectively). Body weight at the end of the experiment did not differ between groups (int+con-graft = $395 \pm$

19 g; NGF-graft = 386 ± 19 g), and mortality rate was equal in the two groups (see *Methods*).

Long-Term Survival of the Grafted Immortalized Progenitors and Persistent Transgene Expression. Nine months postgrafting, after completion of the behavioral tests, ^3H -labeled thymidine autoradiography revealed viable grafts in all animals transplanted with either control or NGF cells (Fig. 2). As observed in previous studies, the grafted stem cells had integrated into the host brain within a radius of approximately 1–1.5 mm around the implantation site, both in the NBM region (Fig. 2A) and in the MS (Fig. 2B), thus covering those regions where the NGF-sensitive basal forebrain cholinergic neurons are located. The grafted cells were found cytoarchitecturally well integrated in the host tissue, some of them in close apposition to the neuronal cell bodies (Fig. 2C).

RT-PCR assessment of expression of the retroviral transcript coding for NGF in tissue samples from the grafted NBM region (Fig. 3) revealed expression of the NGF transgene in two of three rats at 4 months and in three of six rats at 9 months postgrafting. This extends our previous observations, where continued *in vivo* expression of NGF was shown at both the RNA and protein level at 10 weeks after transplantation in the NGF-HiB5 cells (19, 20).

NGF Prevents the Age-Induced Cholinergic Neuron Atrophy. Histological examination of the target neurons detected by immunohistochemistry for the low-affinity neurotrophin receptor (p75^{NTR}) provided data in support of the behavioral differences among int+con-grafted and NGF-grafted animals. The present strain of animals does not undergo any significant reduction in the number of basal forebrain cholinergic neurons with aging, although the animals display a marked cholinergic neuronal atrophy, as assessed with stereological techniques (19, 20). Although the int+con-graft aged animals showed a reduction of cell size of

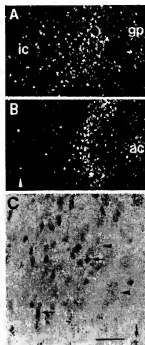


Fig. 2. Survival and integration of transplanted neural stem cells. Dark-field images of autoradiograms of [^3H]thymidine-labeled NGF-secreting cells grafted into the NBM or MS region (A and B). The cells had migrated out from the original implantation site and become integrated in the surrounding brain parenchyma (ic, internal capsule; gp, globus pallidus; ac, anterior commissure; arrowhead in B denotes the midline). (C) A bright field view of a Nissl-stained section at the MS target location, showing grafted cells (coated with silver grains, arrows) intermingled with host neurons (arrowheads). [Bar = 100 μm (A and B) or 15 μm (C).]

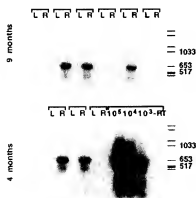


Fig. 3. RT-PCR amplification of the retroviral NGF transcript demonstrates long-term *in vivo* expression in samples from grafted animals. These animals received control- or NGF-secreting neural stem cells in the left (L) or right (R) hemispheres, respectively, and were sacrificed at 4 or 9 months postgrafting. Amplification of the retroviral transcript was performed on RNA extracted from frozen, dissected tissue blocks. The standards were prepared in parallel, by using known amounts of cells (10^3 to 10^5) taken from dividing cultures and mixed with rat brain tissue before RNA isolation. Lane labeled as -RT corresponds to the standard containing the highest amount of NGF cells, subjected to mock reverse transcription (in the absence of reverse transcriptase) and then run in parallel to the other samples for the PCR amplification. Molecular weight standards are Boehringer Type VI.

about 25% in both MS and NBM compared with the 12-month-old adult animals (see Table 1), the NGF-cell-grafted aged animals did not show any sign of neuronal atrophy (6–8% increase above values seen in the adult animals, nonsignificant). In addition, an extensive p75^{NTR} immunoreactive network of processes that did not differ from that normally present in adult animals was present in MS and NBM in the NGF-cell-grafted aged animals but not in the int+con-graft aged rats (Fig. 4).

DISCUSSION

The present results demonstrate that continuous local supply of transgenic NGF from implanted neural progenitors into middle-aged cognitively unimpaired rats can prevent the appearance of age-induced decline in cognitive function as assessed in the spatial navigation task. In previous studies NGF has been administered over a 4- to 10-week period to already impaired aged animals by using either implants of NGF-secreting cells (18–21, 26) or intraventricular infusions of the neurotrophic factor (5–8, 10). These studies had shown that neurotrophic stimulation of either of the two principal basal forebrain cholinergic cell groups (MS and NBM) can reverse cholinergic neuron atrophy and significantly improve the performance of cognitively impaired aged rats in the water maze task. Here we report that similar local, low-level

delivery of NGF, initiated at a presymptomatic stage, can prevent the appearance of both structural and behavioral changes during the subsequent 9 months. Local NGF administration by NGF-secreting neural progenitors did not seem to induce any adverse behavioral side-effects: supply of transgenic NGF to nonimpaired middle-aged rats did not improve the rat's performance above that seen in the nongrafted controls, nor did the NGF cells cause any behavioral impairments in either the water maze or the DMP7 task, which is in contrast to what has previously been reported after implantation of NGF-secreting fibroblasts or direct intracerebral NGF infusions in young subjects (8, 18).

The immortalized neural progenitor cell line used here, Hib5, exhibits highly suitable properties for intracerebral transplantation. In particular, the ability of Hib5 cells to differentiate into glia-like cells and stably integrate into the host brain makes them very well suited for *ex vivo* gene transfer to the CNS. Previous results show that the grafted Hib5 cells may divide two to three times during the first 5 days after implantation; during this time they migrate out from the site of implantation to become fully integrated into the surrounding host tissue within an area with a radius of about 1–1.5 mm. The cell number and distribution attained within the first 2 weeks after grafting remain stable over the subsequent months (22, 28). The *in vivo* expression of the NGF transgene in the NGF-transduced Hib5 cells used here has been examined in some detail. Previous results indicate that NGF-mRNA expression in the grafted cells is reduced by about one order of magnitude within the first 1–2 weeks after grafting but the level of expression then is maintained at a seemingly stable level for at least 10 weeks (19, 22). Consistent with the PCR data, NGF bioassay and NGF ELISA determinations have demonstrated significant bioactive NGF protein levels in the grafted region at both 4 and 10 weeks after transplantation (19, 20). The present results demonstrate that the retroviral vector is still expressed *in vivo* 9 months after transplantation. Comparisons of the present results with previous analyses performed at shorter survival times do not suggest any substantial change in expression between 2 and 9 months postgrafting, although the negative PCR data obtained in some of the present animals (1/3 at 4 months, 3/6 at 9 months) may be taken to indicate that the NGF expression may become more variable at the longest survival times.

The NGF secretion rate of the present NGF-Hib5 transplants can be estimated at about 150 ng/day on each side (see refs. 19, 20, and 22 for further discussion). By bioassay and NGF-ELISA determinations, the grafted cells have been shown to provide a sustained increase in the tissue NGF content in the grafted NBM area, up to the level normally seen in hippocampus or cortex as assessed at 4 and 10 weeks postgrafting (19, 20). Even though the NGF production of the grafted cells may decline in some animals at longer survival times, we propose that the local supply of low levels of the neurotrophic factor from cells located in the immediate vicinity of the cholinergic target neurons, as obtained here, is sufficient to exert a long-lasting neuroprotective effect during aging.

Table 1. Morphometric analysis of p75^{NTR}-immunoreactive neurons

Group	Medial septum		Nucleus basalis	
	Cell counts	Cell volume, μm^3	Cell counts	Cell volume, μm^3
Adult (12 months old)	5,269 \pm 355	7,381 \pm 256	6,126 \pm 271	11,332 \pm 604
Int + con-graft (aged)	5,084 \pm 292	5,554 \pm 463 [†]	6,418 \pm 318	8,640 \pm 293 [‡]
NGF-graft (aged)	4,924 \pm 314	7,847 \pm 662	6,082 \pm 473	12,193 \pm 433

Intact and control-graft groups did not differ in any of the measured parameters ($P > 0.05$). Medial septum: cell counts, intact = 5,451 \pm 684, control-graft = 4,810 \pm 488 cells; volume, intact = 5,524 \pm 1,117 μm^3 , control-graft = 5,576 \pm 356 μm^3 . Nucleus basalis: cell counts, intact = 6,361 \pm 671 cells, control-graft = 6,462 \pm 359 cells; volume, intact = 8,271 \pm 582 μm^3 , control-graft = 8,918 \pm 269 μm^3 .

[†]Different from adult, $P < 0.05$, one-way ANOVA post-hoc Fisher PLSD.

[‡]Different from NGF-graft, $P < 0.05$, one-way ANOVA post-hoc Scheffé *F*-test.

[§]Different from adult and NGF-graft, $P < 0.05$, one-way ANOVA post-hoc Scheffé *F*-test.

The results presented here thus demonstrate the usefulness of the *ex vivo* gene transfer approach for long-term intracerebral delivery of neurotrophic factors, as compared with other alternative methods of administration. Systemically administered neurotrophic factors are not able to cross the blood-brain barrier (BBB), and their diffusion through the brain parenchyma is limited (29), which creates steep concentration gradients when delivered from a point source (30). Indeed, NGF itself has a short half-life (45 min) when delivered to the brain interstitium (31), and its *in vivo* effects disappear after a few days following administration (32). An alternative to intracerebral delivery is the use of conjugates of trophic factors to antibodies (anti-transferrin receptor-NGF) (9, 33) that would make them transported across the BBB by virtue of specific receptors; however, this strategy results in a nontargeted delivery of the trophic factor to all parts of the nervous system (33). Although behavioral effects have been obtained in aged animals by using this approach (9), complications may arise because most neurons in the peripheral nervous system are endowed with (often multiple) sets of neurotrophin receptors that regulate their survival, growth, or function (12, 34). Additionally, outside the nervous system there are other systems that may be responsive to systemically administered neurotrophic factors, as is the case for memory B lymphocytes and NGF (35).

Age-related changes in the forebrain cholinergic system provide a well characterized and highly useful experimental model of progressive neurodegeneration, associated with severe cognitive decline analogous to dementia in man. This study makes use of this model to explore the efficacy of long-term, low-level targeted intracerebral delivery of a neurotrophic factor as a potential therapeutic strategy to prevent the appearance of both morphological changes and behavioral decline associated with advanced

age. The results suggest that targeted gene transfer to vulnerable or affected brain regions can provide interesting new possibilities to achieve long-term delivery of therapeutic proteins within the CNS. Cholinergic neuron atrophy and degeneration is prominent in patients with Alzheimer disease (AD), and although neurodegeneration is widespread in AD, cholinergic neuron dysfunction is believed to play a role in the development of cognitive symptoms, particularly in the early stages of the disease (1). Animal experiments indicate that the basal forebrain cholinergic system is an important modulator of cortical and hippocampal functions and that the subcortical cholinergic afferents play a normal physiological role, e.g., in regulation of attentional processes and short-term spatial or working memory (1, 4). Consistent with this view, it has been shown that selective damage to the basal forebrain cholinergic system (obtained by local injections of the 192IgG-saporin immunotoxin) induces pronounced and long-lasting impairments in the rats' performance in spatial learning and short-term memory tasks; however, these effects are seen only after extensive lesions, which remove >80–85% of the cholinergic neurons in both NBM and the septal-diagonal band area (24, 36, 37). Less extensive lesions, or lesions restricted to either of the two projection systems, have only marginal effects in these tasks (38–40). Because the forebrain cholinergic system in aged rats is largely spared, though in an atrophic state, it seems likely that the age-dependent behavioral impairments may reflect dysfunctions in both cholinergic and noncholinergic systems. The behavioral improvements obtained after intracerebral administration of NGF, therefore, could be due to effects mediated not only by its known neurotrophic actions on the forebrain cholinergic neurons, but also by effects on other systems in the brain (cf. ref. 41).

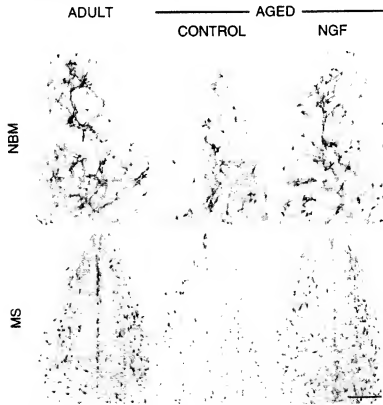


Fig. 4. NGF prevents the age-induced atrophy of forebrain cholinergic neurons. The photomicrographs illustrate p75^{NTR} immunostaining at the level of the NBM (Upper) and MS (Lower) in sections from intact adult or aged animals grafted with control- or NGF-secreting neural stem cells. Note the increased cell body size and intensity of immunostaining of both the neuronal soma and the surrounding network of processes in the NGF-grafted animal. As illustrated in Fig. 2, the grafted cells (not visible here) were cytoarchitecturally integrated in the target region and did not cause any abnormality in the anatomical patterns of the region receiving the graft. (Bar = 200 μ m.)

Although the cellular and behavioral effects of NGF reported here may readily be explained by a direct action of the neurotrophin at the $p75^{NTR}$ and TrkA receptors, both present in forebrain cholinergic neurons, other indirect beneficial actions of NGF should be considered as well. In the brain of both aged individuals and AD patients, inflammation is being recognized as an important component of a pathophysiological mechanism, contributing to both normal aging and the progression of the disease (42, 43). Interestingly, NGF has been shown to modulate excitotoxic processes *in vivo*, leading to a substantial rescue of striatal projection neurons that lack any NGF receptor, through a mechanism that could be related to the control of microglial and glial responses to injury, which are central to the inflammatory process (44, 45). Other possible NGF actions, such as preservation of mitochondrial function against oxidative stress, may be applicable as well (46). It is conceivable, therefore, that a combined trophic and antiinflammatory mechanism may be important for the neuroprotective effects seen in the present experimental paradigm.

In conclusion, the present results provide evidence in support of the hypothesis that sustained local supply of a neurotrophic factor at the cell body level can retard or diminish the normal age-induced progressive degenerative changes in the forebrain cholinergic system to a level where no cellular or behavioral changes can be detected at advanced age. The results show, moreover, that genetically modified neural progenitor cells, by virtue of their integrative properties and stability in the adult or aged brain, are highly useful vehicles for continuous local administration of trophic factors to defined target areas within the CNS. Expression of the high-affinity NGF receptor *trkA* gene is decreased in both AD patients and aged rats, resulting in an impaired retrograde NGF transport in the basal forebrain cholinergic system (47, 48). The *ex vivo* gene transfer of NGF locally, at the cell body level, therefore may be particularly well suited in the context of age-related neuronal atrophy, because it may circumvent those disease-related pathological processes that affect uptake and retrograde transport of the trophic factor from remote target areas. This approach also may have implications for the design of therapeutic strategies for other progressive neurodegenerative conditions, particularly those that are characterized by protracted deterioration of defined subsets of neurons within the CNS.

The authors wish to thank the excellent animal care provided by Sten Nilsson and the expert technical help of Birgit Haraldsson, AnnaKarin Oldén, Gertrude Stridsberg, Kerstin Fogelström, Ulla Jari, Alicia Fläsch, and Cristina Ciornei. Help with image analyses, data handling, and computer editing of the manuscript by Sölveida Cane is gratefully acknowledged. This work was supported by grants from the Swedish Medical Research Council (19X-11632 to A.M.-S. and 04X-3874 to A.B.), the Human Frontier Science Program, the Faculty of Medicine of the University of Lund, and the Segerfalk, Åke Wiberg, and Koek foundations.

- Dunnett, S. B. & Fibiger, H. C. (1993) *Prog. Brain Res.* **98**, 413–420.
- Finch, C. E. (1993) *Trends Neurosci.* **16**, 104–110.
- Gallagher, M. & Colombo, P. (1995) *Curr. Opin. Neurobiol.* **5**, 161–168.
- Everitt, B. J. & Robbins, T. W. (1997) *Annu. Rev. Psychol.* **48**, 649–684.
- Fischer, W., Wictorin, K., Björklund, A., Williams, L. R., Varon, S., & Gage, F. H. (1987) *Nature (London)* **329**, 65–68.
- Fischer, W., Björklund, A., Chen, K., & Gage, F. H. (1991) *J. Neurosci.* **11**, 1889–1906.
- Fischer, W., Sirevaag, A., Wiegand, S. J., Lindsay, R. M., & Björklund, A. (1994) *Proc. Natl. Acad. Sci. USA* **91**, 8607–8611.
- Markowska, A. L., Kolosits, V. E., Breckler, S. J., Price, D. L., & Olton, D. S. (1994) *J. Neurosci.* **14**, 4815–4824.
- Backman, C., Rose, G. M., Hoffer, B. J., Henry, M. A., Bartus, R. T., Friden, P., & Granholm, A. C. (1996) *J. Neurosci.* **16**, 5437–5442.
- Frick, K. M., Price, D. L., Kolosits, V. E., & Markowska, A. L. (1997) *J. Neurosci.* **17**, 2543–2550.
- Lindsay, R. M., Wiegand, S. J., Altar, C. A., & Distefano, P. S. (1994) *Trends Neurosci.* **17**, 182–190.
- Lewin, G. R. & Barde, Y.-A. (1996) *Annu. Rev. Neurosci.* **19**, 289–317.
- Rylett, R. J. & Williams, L. R. (1994) *Trends Neurosci.* **11**, 486–490.
- Hellweg, R., Fischer, W., Hock, W., Gage, F. H., Björklund, A., & Thoenen, H. (1990) *Brain Res.* **537**, 123–130.
- Crutcher, K. A. & Weingartner, K. (1991) *Neurobiol. Aging* **12**, 449–454.
- Williams, L. R. (1991) *Exp. Neurol.* **113**, 31–37.
- Lewin, G. R. & Mendell, L. M. (1993) *Trends Neurosci.* **16**, 353–359.
- Chen, K. S. & Gage, F. H. (1995) *J. Neurosci.* **15**, 2819–2825.
- Martínez-Serrano, A., Fischer, W., & Björklund, A. (1995) *Neuron* **15**, 473–484.
- Martínez-Serrano, A., Fischer, W., Söderström, S., Ebendal, T., & Björklund, A. (1996) *Proc. Natl. Acad. Sci. USA* **93**, 6355–6360.
- Lindner, M. D., Kearns, C. E., Winn, S. R., Frydel, B., & Emerich, D. F. (1996) *Cell Transplant.* **5**, 205–223.
- Martínez-Serrano, A., Lundberg, C., Horellou, P., Fischer, W., Bentlage, C., Campbell, K., McKay, R. D. G., Mallet, J., & Björklund, A. (1995) *J. Neurosci.* **15**, 5668–5680.
- Renfranz, P. J., Cunningham, M. G., & McKay, R. D. G. (1991) *Cell* **66**, 713–729.
- Leanza, G., Muir, J., Nilsson, O. G., Wiley, R. G., Dunnett, S. B., & Björklund, A. (1996) *Eur. J. Neurosci.* **8**, 1535–1544.
- Fischer, W., Chen, K. S., Gage, F. H., & Björklund, A. (1992) *Neurobiol. Aging* **13**, 9–23.
- Winn, S. R., Lindner, M. D., Lee, A., Haggert, G., Francis, J. M., & Emerich, D. F. (1996) *Exp. Neurol.* **140**, 126–138.
- Pelleymounter, M. A., Cullen, M. J., Baker, M. B., Gollub, M., & Wellman, C. (1996) *Mol. Chem. Neuropharmacol.* **29**, 211–226.
- Lundberg, C., Martínez-Serrano, A., Cattaneo, E., McKay, R. D. G., & Björklund, A. (1997) *Exp. Neurol.* **145**, 342–360.
- Yan, Q., Matheson, C., Sun, J., Radec, M. J., Feinstein, S. C., & Miller, J. A. (1994) *Exp. Neurol.* **127**, 23–36.
- Krewson, C. E., Klarman, M. L., & Saltzman, W. M. (1995) *Brain Res.* **680**, 196–206.
- Krewson, C. E. & Saltzman, W. M. (1996) *Brain Res.* **727**, 469–481.
- Krusel, B. N., Kaplan, D. R., & Hefti, F. (1996) *Exp. Neurol.* **139**, 121–130.
- Kordower, J. H., Charles, V., Bayer, R., Bartus, R. T., Putney, S., Walus, L. R., & Friden, P. M. (1994) *Proc. Natl. Acad. Sci. USA* **91**, 9077–9080.
- McMahon, S. B. & Priestley, J. V. (1995) *Curr. Opin. Neurobiol.* **5**, 616–624.
- Torcia, M., Bracci-Laudiero, L., Lucibello, M., Nencioni, L., Lahardi, D., Rubertelli, A., Cozzolino, F., Aloe, L., & Garaci, E. (1996) *Cell* **85**, 345–350.
- Waite, J. J., Chen, A. D., Wardlaw, M. L., Wiley, R. G., Lappi, A. D., & Tal, L. J. (1995) *Neurosci. Res.* **65**, 463–476.
- Leanza, G., Nilsson, O. G., Wiley, R. G., & Björklund, A. (1995) *Eur. J. Neurosci.* **7**, 329–343.
- Torres, E. M., Perry, T. A., Blokland, A., Wilkinson, L. S., Wiley, R. G., Lappi, D. A., & Dunnett, S. B. (1994) *Neuroscience* **63**, 95–122.
- Baxter, M. G., Bucci, D. J., Sobel, T. J., Williams, M. J., Gorman, L. K., & Gallagher, M. (1996) *Neuroreport* **7**, 1417–1420.
- McMahon, R. W., Sobel, T. J., & Baxter, M. G. (1997) *Hippocampus* **7**, 130–136.
- Mervis, R. F., Pope, D., Lewis, R., Dvorak, R. M., & Williams, L. R. (1991) *Ann. N. Y. Acad. Sci.* **640**, 95–101.
- Patterson, P. H. (1995) *Curr. Opin. Neurobiol.* **5**, 642–646.
- Perry, V. H., Bell, M. D., Brown, H. C., & Matyszak, M. K. (1995) *Curr. Opin. Neurobiol.* **5**, 636–641.
- Martínez-Serrano, A., & Björklund, A. (1996) *J. Neurosci.* **16**, 4604–4616.
- Levi-Montalcini, R., Skaper, S. D., Dal Toso, R., Petrelli, L., & Leon, A. (1996) *Trends Neurosci.* **19**, 514–520.
- Galpern, W. R., Matthews, R. T., Beal, M. F., & Isaacson, O. (1996) *Neuroreport* **7**, 2639–2642.
- Cooper, J. D., Lindholm, D., & Sofroniew, M. V. (1994) *Neuroscience* **62**, 625–629.
- Mulson, E. J., Li, J. M., Sobrevieja, T., & Kordower, J. H. (1996) *Neuroreport* **8**, 25–29.

Long-term functional recovery from age-induced spatial memory impairments by nerve growth factor gene transfer to the rat basal forebrain

(experimental gene therapy/immortalized neural stem cells/learning and memory/aging/neurotrophins)

ALBERTO MARTÍNEZ-SERRANO^{*†}, WALTER FISCHER^{*}, STINE SÖDERSTRÖM[‡], TED EBENDAL[‡], AND ANDERS BJÖRKLUND^{*}

^{*}Department of Physiology and Neuroscience, Lund University, Sölvegatan 17, S-223 62-Lund, Sweden; and [‡]Department of Developmental Neuroscience, Biomedical Center, Box 587, Uppsala University, S-751 23-Uppsala, Sweden

Communicated by Tomas Hökfelt, Karolinska Institutet, Stockholm, Sweden, February 6, 1996 (received for review September 22, 1995)

ABSTRACT Nerve growth factor (NGF) stimulates functional recovery from cognitive impairments associated with aging, either when administered as a purified protein or by means of gene transfer to the basal forebrain. Because gene transfer procedures need to be tested in long-term experimental paradigms to assess their *in vivo* efficiency, we have used *ex vivo* experimental gene therapy to provide local delivery of NGF to the aged rat brain over a period of 2.5 months by transplanting immortalized central nervous system-derived neural stem cells genetically engineered to secrete NGF. By grafting them at two independent locations in the basal forebrain, medial septum and nucleus basalis magnocellularis, we show that functional recovery as assessed in the Morris water maze can be achieved by neurotrophic stimulation of any of these cholinergic cell groups. Moreover, the cholinergic neurons in the grafted regions showed a hypertrophic response resulting in a reversal of the age-associated atrophy seen in the learning-impaired aged control rats. Long-term expression of the transgene lead to an increased NGF tissue content (as determined by NGF-ELISA) in the transplanted regions up to at least 10 weeks after grafting. We conclude that the gene transfer procedure used here is efficient to provide the brain with a long-lasting local supply of exogenous NGF, induces long-term functional recovery of cognitive functions, and that independent trophic stimulation of the medial septum or nucleus basalis magnocellularis has similar consequences at the behavioral level.

Aging in rodents is associated with a progressive decline in learning abilities, memory storage and use of spatial information (1–4), and associated with a hypofunction of the cholinergic forebrain system, with the neurons in the medial septum (MS) and nucleus basalis magnocellularis (NBM) region being most affected and displaying a marked degenerative atrophy (2, 4–11). Memory impairments correlate with cholinergic atrophy in forebrain nuclei (7, 10, 12, 13), and both can be reversed by the exogenous administration of nerve growth factor (NGF) or neurotrophin-3 (NT-3) (6, 9, 14–22). Conversely, administration of anti-NGF antibodies induces memory impairments and cholinergic neuron atrophy in the adult brain (23, 24). The similarities between the age-associated changes in the cholinergic system seen in rodents and the neurodegeneration that occurs in normal aging and in Alzheimer disease in humans (25–30) have raised the possibility that exogenous supply of NGF to the aged brain could be effective as a clinical therapy for the amelioration of cognitive impairments and atrophic neuronal changes. However, the identification of optimal routes for long-term administration

of NGF or other therapeutic proteins to the central nervous system remains a major challenge (31–33). Continuous intracerebral infusion of NGF delivered from minipumps is of limited duration and requires a permanent cannula implantation, a procedure that causes chronic trauma and inflammation. NGF can be made available to the brain after conjugation to other proteins that can be recognized at the blood brain barrier and transported to the brain side (34, 35); this approach, though proven useful, results in a nonlocal NGF supply, generally in a low dose range and of very limited time-duration (35).

Cell-based gene transfer represents an interesting alternative to these procedures for long-term intracerebral delivery of therapeutically active proteins in that it may circumvent the drawbacks associated with chronic intracerebral infusions or injections. Fibroblastic cell lines (encapsulated in some cases), primary fibroblasts, and central nervous system-derived neural progenitors have been used to deliver NGF to the rodent or primate brain, and have been shown to exert neurotrophic effects on lesioned cholinergic neurons of the basal forebrain (36–41) and, in rodents, recovery from age-related cognitive impairments (14, 19). For any gene transfer procedure, however, it is important to demonstrate that the functional effects are long-lasting and that it allows for stable *in vivo* transgene expression that leads to increased levels of the transgenic protein in the brain. The present study was undertaken to examine these two essential aspects of NGF gene transfer to the aged rat brain after transplantation of a clonal neural stem cell line genetically engineered to synthesize and secrete NGF (14, 36). We report here that this *ex vivo* gene transfer approach can be used to ameliorate behavioral deficits in rats for extended time periods, up to 2.5 months, that it is useful in animals of advanced age, and that it provides the target region with an increased amount of NGF protein (as determined by ELISA). Furthermore, we show that local, independent stimulation of cholinergic neurons in the MS and NBM is sufficient to produce a sustained improvement in the ability of the animals to perform the task.

MATERIALS AND METHODS

Gene Transfer. *Ex vivo* gene transfer of NGF was performed by transplanting control (clone D11) or NGF-secreting (clone E8) conditionally immortalized neural progenitors as described (36). A brain-derived neurotrophic factor (BDNF)-producing clone (C7) engineered in the same way to secrete hBDNF (h, human) (42) was used in one group of animals to

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: MS, medial septum; NBM, nucleus basalis magnocellularis; PS, postsurgery; NGF, nerve growth factor; RT-PCR, reverse transcription-PCR; BDNF, brain-derived neurotrophic factor.

†To whom reprint requests should be addressed. e-mail: abto@biogen.wlab.lu.se.

control for specificity of the neurotrophic effects. A detailed characterization of these neural progenitor cell lines *in vitro* and *in vivo* after transplantation can be found elsewhere (14, 36, 42, 43). Cells were cultured in DMEM containing 10% fetal bovine serum, 2 mM glutamine, and 10,000 units per ml of both penicillin and streptomycin. For transplantation, cell suspensions in Hanks' balanced salt solution (GIBCO) at 100,000 cells per μ l were prepared by trypsinization and used within 3 hr. The cells were labeled for 72 hr before grafting with [3 H]thymidine [10μ Ci/ml (1 Ci = 37 GBq), Amersham].

Animal Groups and Surgical Procedures. The animals used in this study were 18 young (3 months old) and 54 aged female Sprague-Dawley rats that were housed and treated following institutional guidelines. Before grafting, 10 young rats and 90 aged animals (24 months old) were tested in the Morris water maze (15). Ten aged rats were classified as nonimpaired in the task, and 35 aged impaired animals were further subdivided into the following groups: Control (receiving a graft of D11 cells into the septum, $n = 6$, or NBM, $n = 5$), NGF cells in MS (grafted with E8 cells bilaterally into the MS, $n = 9$), NGF cells in NBM (grafted with E8 cells bilaterally into the NBM region, $n = 7$), and BDNF cells in the NBM (grafted with C7 cells bilaterally into the NBM region, $n = 8$). Coordinates for the different transplants were as follows: NBM: (TB + 5.0) AP = +0.2, ML = ± 3.4 , V(d) = -7.0 and AP = +1.0, ML = ± 2.6 , V(d) = -7.3; MS: (TB - 2.0) AP = +0.5, ML = ± 0.6 , V(d) = -7.0 and -6.7. Four deposits (two on each side) were placed in either the MS or in the NBM region; a total of 400,000 cells (100,000 cells in 1 μ l per deposit) were injected in each rat. Because the animals grafted with control cells in the MS or NBM regions did not differ during behavioral testing, they are treated as a single combined control-graft group. The aged animals that were used to demonstrate long-term expression of the transgene [13 rats for NGF ELISA measurements and 4 rats for reverse transcription-PCR (RT-PCR) amplification of the retroviral transcript] were either rats that did not fit the criteria for inclusion into the nonimpaired or impaired groups, or animals with a swimming speed lower than 0.11 m/s (the only parameter used to exclude animals from the test). Eight young rats bilaterally grafted with control- or NGF-cells in the NBM in different hemispheres were used for NGF-ELISA determination from tissue samples. All animals were sacrificed 10 weeks after grafting once the behavioral experiment was completed; out of 54 transplanted aged rats, only four rats died during the experiment (from 25 to 27–28 months old).

Long-Term Behavioral Test. Animals were tested for spatial learning and memory in the Morris water maze test (44) and were given daily blocks of eight trials with a cut-off time of 60 s. The tests were arranged as follows (see Fig. 1): pretest, used to allocate the animals to the different experimental groups during a 5-day testing schedule; test 1, starting 8 days post-surgery (PS) consisting of 5 days of testing; test 2, performed 7 weeks after grafting with a duration of 3 days; test 3, starting the day after the finish of test 2 (day 53 PS) with a duration of 16 days (3 days of testing, 10 days of rest, and another 3 days of testing); visible platform test was performed on the day after test 3 and lasted 1 day (day 71 PS).

The submerged platform was located in the southwestern quadrant of the pool during the pretest, test 1, and test 2, and it was changed to the northeastern quadrant for test 3 to study the ability of the rats to learn the task. Spatial probe trials (removed platform) were given at the end of day 5 in the pretest and test 1, and at the end of days 3 and 16 during test 3.

Analyses of Transgene Expression *In Vivo*. Ten weeks after grafting (day 72 PS), the animals were killed under halothane anesthesia, and their brains were quickly removed and frozen under crushed dry ice; the selected regions were dissected at subzero temperatures. RT-PCR amplification of the NGF-coding retroviral transcript was performed on total RNA extracted from these pieces of grafted tissue (14, 36). NGF

ELISA assay was applied in parallel tissue samples following described procedures (45) using a monoclonal anti-mouse β -NGF antibody 27/21 as a capture antibody and a sandwich 27/21- β -galactosidase-conjugate antibody (Boehringer Mannheim).

Histology. Animals that participated in the behavior experiment (together with the young control subjects) were perfused at the end of the experiment with buffered 4% paraformaldehyde and their brains were sectioned at 40 μ m thickness; [3 H]thymidine autoradiography, immunohistochemical staining for the low-affinity Neurotrophin receptor (p75^{NTR}), and stereological assessment of cell numbers and volumes in the MS and NBM regions were performed following standard procedures (14, 36, 46).

RESULTS

Long-Term Recovery from Age-Associated Learning and Memory Impairments. Before grafting, the aged animals were tested in parallel with a group of young (3 months old) rats and classified as nonimpaired or learning impaired on the basis of previously defined criteria (ref. 15; escape latency for the nonimpaired rats was within the mean \pm two standard deviations of the young group during the last 2 days of the test) (Fig. 1). Starting on day 8 PS, the animals were tested for 5 days in the water maze for their spatial navigation performance; during this week (test 1), overall, the NGF-grafted rats performed better than the control-grafted animals (Fig. 1). Both NGF-grafted groups were not different from the nonimpaired rats, whereas the control-grafted animals remained impaired. The animals transplanted in the MS were significantly better than the controls at the beginning of the test, and the NBM-grafted rats showed a nonsignificant trend to reduced escape latency in this initial test. Eight weeks after grafting, during test 2 (starting on day 50 PS) and regardless of the location of the transplant (MS or NBM), the NGF-grafted animals showed a further reduction in the time to find the platform and were indistinguishable from the nonimpaired animals, and significantly different from the control-graft group.

In the subsequent test 3, the platform was moved to a new location to study the rats ability to learn the task; after this change, all groups showed an increase in the escape latency parameter (repeated measures ANOVA, $F_{1,30} = 37.1$, $P = 0.0001$, effect of day, day 3 of test 2 versus day 1 of test 3), indicating that the rats were using a spatial strategy to find the platform at the end of test 2. In test 3, all groups reduced their escape latency scores to the level seen at the end of test 2 ($P = 0.0003$, $F_{1,30} = 17$, for repeated measures), showing that they could learn the reversal task using new spatial extra maze cues.

The animals were also tested for their ability to remember the location of the hidden platform in the spatial probe trials (removed platform), performed at the end of the pretest, test 1 and twice during test 3 (Table 1). Both groups of NGF-transplanted rats had a score as low as the control-grafted animals in the pretest, but improved their spatial acuity (number of crossings over the former platform site, indicating focused search) significantly during test 3 in two different trials (days 3 and 16 of test 3), demonstrating that the NGF-grafted groups had improved their ability to remember the platform location. In the first day of test 3, when the platform location was changed, the nonimpaired and both NGF-grafted groups showed during the first swim a marked focused search, resulting in a higher number of crossings over the former platform location (Table 1). In the spatial probe trials during test 3 (analyzed as the average of the trials at days 3 and 16), the nonimpaired rats and both NGF-groups, but not the impaired control animals, displayed significant focus over the new platform site, indicating that the improved spatial learning was maintained for at least 10 weeks in the rats receiving NGF-cells. The same results were obtained when analyzing swim distances, and, consistent with this, the swimming speeds did

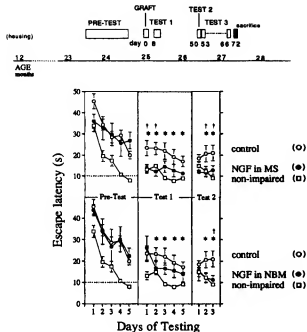


Fig. 1. Spatial memory testing. (Upper) Time course of the experiment and subjects age during the testing; the indicated days PS refer to the start of each test. (Lower) Nonimpaired rats (□), animals grafted with control-cells (○), and two groups of animals grafted with NGF-cells (●) in the MS (upper plots) or NBM region (lower plots) were tested for spatial navigation in the water maze. The diagram shows the escape latency scores to find the platform during the pretest session (pretest) and two postgrafting tests, performed during the second (test 1) or eighth week (test 2) after surgery. During the pretest, the nonimpaired group was significantly different ($P < 0.05$) from the other groups during days 2, 4, and 5. Young animals tested in parallel did not differ from the nonimpaired group at any day during the pretest (the performance reached by the young group on the 5th day, 10 ± 1.3 s, is shown for comparison as a dotted line). After grafting, the control-group was still different from the nonimpaired group and the NGF-groups improved their performance to the extent that they were no longer different from the nonimpaired group (*) on the days indicated by (†). The NGF groups were significantly different from the control-graft group and not from the nonimpaired ($P < 0.05$, one-way ANOVA, post hoc Fisher pairwise least significant difference).

not differ among groups during the experiment. Moreover, in the visible platform test, there were no differences in escape latency among groups ($F_{3,30} = 1.01$, $P = 0.404$, one-way ANOVA). Thus, the improved task performance seen in the rats receiving grafts of NGF-secreting cells could not be ascribed to differences in motor performance.

An additional group of animals was grafted in the NBM region with a similar cell line engineered to secrete BDNF (42); this BDNF-graft group did not show any improvement in any parameter during the testing, being in all cases similar to the control group (data not shown).

MS Versus NBM Location of the NGF-Producing Cell Grafts. Throughout testing, no clear-cut differences were found between the animals transplanted in the MS or the NBM region in either escape latency or the number of crossings over the former platform site. During test 3, the number of crossings in the spatial probe trial showed that both NGF-grafted groups were clearly improved compared with the control-grafted rats, and they were not different from the nonimpaired animals (Table 1). However, the NGF-grafted groups were slightly different from each other in the escape latency parameter during the relearning (test 3, after changing the location of the platform). Thus, the NBM-graft group performed similarly to

Table 1. Platform crossings in spatial probe trials

	Pre test	Test 1	Test 3*	Test 3 ^b
Nonimpaired ^c	$3.8 \pm 0.3^*$	$5.1 \pm 0.4^*$	$3.6 \pm 0.7^*$	$4.2 \pm 0.5^*$
Control MS and NBM	$2.2 \pm 0.4^†$	$3.1 \pm 0.7^†$	$1.1 \pm 0.4^†$	$1.5 \pm 0.3^†$
NGF-MS	$1.6 \pm 0.3^†$	$2.6 \pm 0.5^†$	$3.2 \pm 0.6^*$	$4.1 \pm 0.5^*$
NGF-NBM	$1.4 \pm 0.5^†$	$5.4 \pm 0.9^*$	$3.8 \pm 1.0^*$	$3.0 \pm 0.6^*$

Different from control-graft (*) or nonimpaired (†), $P < 0.05$, one-way ANOVA, Fisher PLSD post hoc test. Values are given as mean \pm SEM.

*Number of crossings over the southwestern location (used as platform site in previous tests), once the platform was shifted to northeastern for test 3.

^bAverage of crossings during the 3rd and 16th days of test 3.

^cNontransplanted animals.

the control group, whereas the MS-graft group was indistinguishable from the nonimpaired rats (data not shown).

Long-Term *In Vivo* Expression of the Transgene. NGF protein was quantified in tissue pieces dissected from animals grafted in the NBM with control- or NGF-cells and compared with other brain regions; the hippocampus and cortex were used as high-NGF content controls and ventral mesencephalon as low NGF-content tissue. The amount of immune-detectable NGF protein in an ELISA assay is shown in Table 2, both for young (4 weeks after grafting) and aged animals (10 weeks after grafting). The NGF levels in the NGF-grafted regions were clearly higher than in the control-grafted regions (increasing 1.55- and 2.64-fold in young and aged rats, respectively, $P = 0.01$ in both cases). Compared with the NGF content in the cortex (100% at each age), the NGF values recorded in the NGF-grafted NBM amounted to 84% and 122% in the young and aged recipients, respectively, whereas the values in the control-grafted NBM were only 54% and 46%, respectively. Consistent with the NGF-ELISA data, RT-PCR amplification of the retroviral transcript in total RNA samples from grafted NBM tissue revealed clear expression of the NGF transgene in four aged animals analyzed at 10 weeks after transplantation (data not shown).

Histological Analyses. [³H]Thymidine autoradiography revealed surviving grafts in all grafted animals (Fig. 2D and H): The grafted cells were found scattered around the implantation sites, covering a distance of about 1 mm from the sites of injection, with a glia-like morphology in Nissl-stained sections. The morphological appearance, migration, and integration of the control- and NGF-cells were similar to those described earlier at 10 weeks after transplantation in young animals and at 4 weeks after transplantation in aged rats (14, 36).

NGF-producing grafts induced a hypertrophic response of the p75^{NTR}-positive neurons in each target region (163% and 182% of control-graft values in the MS and NBM, respectively; seen only when the NGF graft was placed in these regions; four to seven randomly selected animals per group; Fig. 2 and Table 3). By contrast, grafts of BDNF-producing cells had no effect. In the NBM, the NGF-induced local trophic response led to a normalization of cell volumes in the aged animals compared

Table 2. NGF ELISA determination on tissue samples from transplanted adult or aged animals at 4 or 10 weeks after grafting

Region	NGF content (pg/g wet tissue)	
	Young (n = 8)	Aged (n = 13)
Ventral mesencephalon	189 ± 24	203 ± 49
Hippocampus	1929 ± 338	1503 ± 120
Cortex	709 ± 55	690 ± 79
NBM, control-graft	$382 \pm 57^*$	$320 \pm 59^*$
NBM, NGF-graft	$592 \pm 86^†$	$845 \pm 178^†$

*Different from cortex, $P < 0.05$ (one-tailed, student's *t* test).

[†]NGF-grafted NBM is different from control-grafted NBM ($P = 0.01$) and not from cortex ($P > 0.4$).

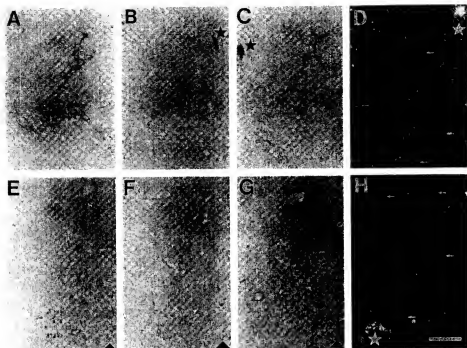


FIG. 2. Reversal of cholinergic neuron atrophy in aged rats by NGF-producing grafts. Overview of p75^{NTR} immunostained cholinergic neurons in the NBM (A–D) and MS (E–H) in nonimpaired (A and E) or memory-impaired animals receiving either control- (B and F) or NGF-secreting (C and G) grafts. The stars denote the original placement of one of the two deposits in each case, dorsal and lateral to the NBM nucleus (A–D) and the arrowheads point to the midline (E–H). (D and H) Darkfield microphotographs of [3H]thymidine autoradiographs showing the NGF-grafts into the NBM (D) and MS (H). Individually labeled grafted cells (identified by grain clusters, white arrows) have integrated into the host tissue within a region extending about 1 mm from the injection site. The stars denote portions of the original implantation site. (Bar in H: A–C, 400 μ m; E and F, 200 μ m, D and H, 100 μ m.)

with both the young and the nonimpaired aged subjects (Fig. 2 A–C). The MS graft group exhibited hypertrophy when compared to the young controls (illustrated in Fig. 2 E–G; Table 3). p75^{NTR}-positive neuron cell counts did not differ between any of the groups (Table 3).

DISCUSSION

The experimental evidence described here demonstrates the long-term efficiency of an NGF-secreting neural stem cell line for NGF gene transfer to the aged rat brain. The results provide evidence for functional recovery of memory-impaired animals after supplying NGF to the relevant cholinergic neuronal populations in MS and NBM, accompanied by a reversal of their atrophic state. *In vivo* transgene expression was

demonstrated by RT-PCR at 10 weeks after gene transfer; moreover, the NGF content in the transplanted regions was increased as determined by ELISA, thus substantiating the sustained *in vivo* production of the transgene protein product from the transplanted engineered cells.

We and others (14, 19) have previously reported ameliorative effects of short-term NGF gene transfer on age-induced memory impairments in rodents in experiments limited to a 3-week test schedule as previously used in NGF infusion experiments (6, 9). In fact, the present study is the first to report sustained effects of any type of exogenous NGF administration in aged rats beyond 1 month, showing that NGF-producing neural stem cells implanted into either of the two principal cholinergic basal forebrain cell groups, MS and NBM, are effective in inducing long-lasting improvement in

Table 3. Morphometric analyses of the p75^{NTR}-positive neurons

	n	MS*		NBM*	
		Number	Volume	Number	Volume
Young ^c	4	22242 \pm 4065	6050 \pm 177	6998 \pm 393	9024 \pm 152
Nonimpaired ^c	5	19885 \pm 1838	6333 \pm 602	5706 \pm 573	9808 \pm 1124
Control MS and NBM	8	19251 \pm 1566	5163 \pm 213*	5748 \pm 560	6294 \pm 162*
NGF-MS	7	17389 \pm 1723	8396 \pm 157*†	6200 \pm 200	6476 \pm 363*
NGF-NBM	4	18525 \pm 2931	5213 \pm 103	5869 \pm 556	11444 \pm 1725†
BDNF-NBM	5	16446 \pm 921	5250 \pm 118	5971 \pm 708	6104 \pm 287*

Cell number and neuronal volume (μ m³) in the MS and NBM regions, ten weeks after grafting control-, NGF-, or BDNF-cells. Values are mean \pm SEM.

*The MS was analyzed bilaterally as a single structure.

†NBM figures from each hemisphere were averaged to obtain cell counts and volumes in each animal.

^cNongrafted groups.

*Different from young and nonimpaired groups.

†Different from control-graft group. *P* < 0.05, one-way ANOVA, Fisher PLSD *post hoc* test. Cell numbers were not different among groups. Control-MS and control-NBM were not different from each other and thus combined in a single control-MS and NBM group.

spatial learning (both escape latency and platform crossings) in the Morris water-maze task for at least 10 weeks after cell implantation. On the platform crossings measure, the most robust index of spatial acuity, both NGF-grafted groups were significantly improved compared with the control group, with scores as high as the nonimpaired animals. Consistent with previous observations using osmotic minipump infusions of BDNF (16), the BDNF-producing cells did not induce any improvement of the rats' performance in this task.

The extent of behavioral improvement found in the present experiment is quite similar to that observed after continuous infusions of high doses of NGF (6 μ g per day for 4 weeks) (refs. 16 and 17; see also ref. 18), and in previous studies on the short-term effects of NGF gene transfer in aged rats (14, 19). Both the acquisition of place navigation (shown as a reduction in escape latency or swimming distance) and the formation/retention of spatial memory (evaluated as platform crossings in the spatial probe trials) were improved. In the present experiment, the long-term stability of the recovery in spatial learning and memory was observed in a 10-week testing paradigm. In particular, the use of *ex vivo* gene transfer procedures allowed for the first time to study the effect of local sustained NGF-delivery on the rats' ability to relearn the task in a long-term experimental design. In the platform reversal test (test 3), the NGF-grafted animals could relearn and retain the new platform location similarly to the nonimpaired rats, whereas the control animals were impaired on the acquisition of this new platform site. These observations imply that the spatial learning abilities of the NGF-grafted rats were improved in a sustained manner, rendering the animals able to make proper use of spatial information to adopt a new spatial search strategy.

Improved spatial learning has previously been observed with NGF-secreting stem cells grafted either into the NBM alone or into both the NBM and MS (14); NGF-secreting fibroblasts have been shown to induce similar effects when transplanted in the NBM region of learning-impaired aged rats (19). In our previous study, we could not elucidate the relative importance of the MS and NBM regions in mediating the NGF-effect. Previous infusion experiments are equally inconclusive in this respect, because intraventricularly administered NGF is widely distributed in the brain and may affect multiple targets, including the basal forebrain, striatum, hippocampus, and cortex (47, 48). In the present work, we confirm that long-term stimulation of the NBM region results in improved performance in the water maze, but we also show that NGF supply confined to the septal area results in an equal or even greater improvement compared with NBM transplants. Interestingly, in young animals, widespread cholinergic deficits induced by the cholinergic immunotoxin 192 IgG-saporin are required to impair task performance in the water maze test, whereas focal MS or NBM lesions have little or no effect, suggesting that each of the two cholinergic nuclei is able by itself to ensure performance in the task (49–51).

Consistent with the pattern of behavioral improvement, the histological analyses revealed a trophic response in the basal forebrain cholinergic neurons only in those animals and regions that received an NGF-producing graft, such that the p75^{NTR}-positive neuron volume was restored or even increased above that found in the young controls. No indication of increased cell numbers was obtained in any of the groups, which is in agreement with our previous short-term gene transfer experiment (ref. 14; see ref. 19), as well as with several NGF infusion experiments (9, 16, 17). The fact that a localized host trophic response, as seen here, is linked to significant spatial memory improvement provides strong evidence in favor of the interpretation that functional stimulation of either of two principal cholinergic forebrain projection systems, i.e., MS or NBM, is sufficient for restoration of spatial memory performance in aged rats, and moreover that local application

of the neurotrophin at the cell body level is sufficient to induce this effect.

The estimated NGF secretion rate of the present NGF grafts (100 ng per day on each side; see ref. 36) is much lower than the amount of NGF necessary to induce similar effects after intraventricular infusion (6 μ g per day). This indicates that the cellular gene transfer system is a considerably more efficient delivery system for neurotrophic proteins than pump infusion into the cerebrospinal fluid. In this context, it should be noted that the *in vitro* NGF secretion rate of the E8 stem cell clone used here is \sim 10-fold higher than that of the transduced primary fibroblasts used by Chen and Gage (19), which may explain why more pronounced cellular and behavioral effects are obtained with the NGF-secreting neural stem cells.

An important aspect addressed in our study relates to the effectiveness of the present *ex vivo* gene transfer approach in providing the brain with a significant amount of transgenic protein, which is a critical point in the validation of any gene transfer protocol. Previous reports (14, 36) have indicated that gene expression from the (monocistronic) retroviral vector in the NGF-stem cell line could be detected *in vivo* as long as 10 weeks in adult animals and 4 weeks in aged subjects, as measured by RT-PCR or as NGF-like neurotrophic activity (14). Here we have used NGF-ELISA determinations on tissue samples dissected from transplanted young or aged rat brains to demonstrate the presence of NGF protein in the grafted NBM region at 4 and 10 weeks after transplantation of the transduced cells, as well as RT-PCR determinations to demonstrate the expression of the retroviral NGF message. The actual quantification of NGF protein at the target region provides a direct demonstration that the cell line used in our studies continues to express the transgene, and that this results in an increased NGF tissue content in the transplanted area. The NGF levels obtained *in vivo* are high enough to exert a wide-spread neurotrophic effect within the target nuclei. Indeed, recent evidence indicates that the continued presence of elevated NGF levels is required to maintain NGF-induced cholinergic neuronal hypertrophy over time. Thus, Kordower *et al.* (52) have reported that the NGF-induced cellular response dissipates within 3 weeks following cessation of exogenous NGF supply.

The results reported here demonstrate the efficiency of the present *ex vivo* gene transfer strategy as a localized delivery system for neurotrophic factors to the brain. When compared with other protein delivery systems to the brain, the current approach shows clear advantages over injections, infusions, or other cellular vehicles, exemplified by fibroblasts: Immortalized neural stem cells provide the brain with an even but targeted source of transgenic protein for long periods of time, resulting in an increased content of the transferred trophic factor. Taken together, available data on the molecular, cellular, and behavioral levels provide a solid basis for the use of neurotrophin-secreting neural stem cell lines as a powerful experimental therapeutic approach to counteract neurodegenerative changes and promote functional recovery in the diseased or injured central nervous system.

The authors wish to thank Sten Nilsson for excellent animal care, Birgit Haraldsson, Anna Karin Olden, Gertrude Stridsberg, Kerstin Fogelström, Alicja Flasch, Ulla Jari, and Cristina Ciorniei for expert technical assistance, and Soledad Conte for data processing. This work was funded by grants from the Human Frontier Science Program (A.B.), the Swedish Medical Research Council (Grant 04X-3874 to A.B. and Grant 19X-11632 to A.M.-S.), the Swedish Natural Science Research Council (Grant B-AA/BU 04024-317 to T.E.), and the Segerfalk, Åke Wiberg and Kock foundations. A.M.-S. was partially supported by the Human Frontier Science Program and the Swedish Medical Research Council.

2. Dunnett, S. B. & Fibiger, H. C. (1993) *Prog. Brain Res.* **98**, 153–420.
3. Finch, C. E. (1993) *Trends Neurosci.* **16**, 104–110.
4. Gallagher, M. G. & Colombo, P. J. (1995) *Curr. Opin. Neurobiol.* **5**, 161–168.
5. Bieganski, A., Greenberger, V., & Segal, M. (1986) *Neurobiol. Aging* **7**, 215–217.
6. Fischer, W., Victorin, K., Björklund, A., Williams, L. R., Varon, S., & Gage, F. H. (1987) *Nature (London)* **329**, 65–68.
7. Fischer, W., Gage, F. H. & Björklund, A. (1989) *Eur. J. Neurosci.* **1**, 34–45.
8. Alaravista, M. C., Bentivoglio, A. A. R., Crociani, P., Rossi, P., & Albanese, A. (1989) *Brain Res.* **455**, 177–181.
9. Fischer, W., Björklund, A., Chen, K. S., & Gage, F. H. (1991) *J. Neurosci.* **11**, 1889–1906.
10. Fischer, W., Chen, K. S., Gage, F. H. & Björklund, A. (1991) *Neurobiol. Aging* **13**, 9–23.
11. Smith, M. L. & Booze, R. M. (1995) *Neuroscience* **67**, 679–688.
12. Koh, S., Chang, P., Collier, T. J., & Loy, T. (1989) *Brain Res.* **498**, 397–404.
13. Gallagher, M., Burwell, R. D., Kodsi, M. H., McKinney, M., Southerland, S., Vella-Roundtree, L., & Lewis, M. H. (1990) *Neurobiol. Aging* **11**, 507–514.
14. Martínez-Serrano, A., Fischer, W. & Björklund, A. (1995) *Neuron* **15**, 473–484.
15. Gage, F. H., Kelly, P. A. T. & Björklund, A. (1984) *J. Neurosci.* **4**, 2856–2865.
16. Fischer, W., Sirevaag, A., Wiegand, S. J., Lindsay, R. M., & Björklund, A. (1994) *Proc. Natl. Acad. Sci. USA* **91**, 8607–8611.
17. Fischer, W. (1994) *Neurochem. Int.* **25**, 47–52.
18. Markowska, A. L., Kollasos, V. E., Breckler, S. J., Price, D. L. & Olson, D. S. (1994) *J. Neurosci.* **14**, 4815–4824.
19. Chen, K. S. & Gage, F. H. (1995) *J. Neurosci.* **15**, 2819–2825.
20. Rylett, R. J., Goddard, S., Schmidt, B. M., & Williams, L. R. (1993) *J. Neurosci.* **13**, 3956–3963.
21. Lindsay, R. M., Wiegand, S. J., Altar, C. A., & DiStefano, P. S. (1994) *Trends Neurosci.* **17**, 182–190.
22. Rylett, R. J. & Williams, L. R. (1994) *Trend Neurosci.* **17**, 486–490.
23. Nitta, A., Murase, K., Furukawa, Y., Hayashi, K., Hasegawa, T., & Nabeshima, T. (1993) *Neuroscience* **57**, 495–499.
24. Van der Zee, C. E. E. M., Lourenssen, S., Stanisz, J., & Diamond, J. (1995) *Eur. J. Neurosci.* **7**, 160–168.
25. Bartus, R. T., Dean, R. L., Beer, B., & Lipka, A. S. (1982) *Science* **217**, 408–417.
26. Coyle, J. T., Price, D. L. & DeLong, M. R. (1983) *Science* **219**, 1184–1190.
27. Whitehouse, P. J., Price, D. L., Struble, R. G., Clark, A. W., Coyle, J. T., & DeLong, M. R. (1982) *Science* **215**, 1237–1239.
28. Mufson, E. J., Bothwell, M., & Kordower, J. H. (1989) *Exp. Neurol.* **105**, 21–232.
29. Wilcock, G. K., Eseri, M. M., Bowen, D. M., & Smith, C. T. (1982) *J. Neurol. Sci.* **57**, 407–417.
30. Strada, O., Vyas, S., Hirsch, E. C., Ruberg, M., Brice, A., Agid, Y., & Javoy-Agid, F. (1992) *Proc. Natl. Acad. Sci. USA* **89**, 9549–9553.
31. Longo, F. M., Holtzman, D. M., Grimes, M. L., & Mobley, W. C. (1993) in *Neurotrophic Factors*, eds. Loughlin, S. E. & Fallon, J. H. (Academic, London), pp. 209–256.
32. Jelsma, T. N. & Aguayo, A. J. (1994) *Curr. Opin. Neurobiol.* **4**, 717–725.
33. Björklund, A. (1993) *Nature (London)* **362**, 154–155.
34. Friden, P. M., Walus, L. R., Watson, P., Doctrow, S. R., Kozarich, J. W., Beckman, C., Begman, H., Hoffer, B., Bloom, F., & Granholm, A. C. (1993) *Science* **259**, 373–377.
35. Kordower, J. H., Charles, V., Bayer, R., Bartus, R. T., Putney, S., Walus, L. R., & Friden, P. M. (1994) *Proc. Natl. Acad. Sci. USA* **91**, 9077–9080.
36. Martínez-Serrano, A., Lundberg, C., Horellou, P., Fischer, W., Bentlage, C., Campbell, K., McKay, R. D. G., Mallet, J., & Björklund, A. (1995) *J. Neurosci.* **15**, 5668–5680.
37. Rosenberg, M. B., Friedmann, T., Robertson, R. C., Tuszyński, M., Wolf, J. A., Breakfield, X. O., & Gage, F. H. (1988) *Science* **242**, 1575–1578.
38. Strömberg, I., Wetmore, C. J., Ebendal, T., Ernfors, P., & Olson, L. (1990) *J. Neurosci.* **10**, 405–411.
39. Kawaja, M. D., Rosenberg, M. B., Yoshida, K., & Gage, F. H. (1992) *J. Neurosci.* **12**, 2849–2864.
40. Dekker, A. J., Fagan, A. M., Gage, F. H., & Thal, L. J. (1994) *Brain Res.* **639**, 149–155.
41. Emerich, D. F., Hammang, J. P., Baetge, E. E., & Winn, S. R. (1994) *Exp. Neurol.* **130**, 115–150.
42. Martínez-Serrano, A., Hantzopoulos, P. A., & Björklund, A. (1996) *Eur. J. Neurosci.* **8**, 727–735.
43. Renfranz, P. J., Cunningham, M. G., & McKay, R. D. G. (1991) *Cell* **66**, 713–729.
44. Morris, R. G. M. (1981) *Lear. Mot.* **12**, 239–260.
45. Söderström, S., Hallböök, F., Ibáñez, C. F., Persson, H., & Ebendal, T. (1990) *J. Neurosci. Res.* **27**, 665–677.
46. Gundersen, H. J. G., Brendsen, T. F., Korbo, L., Marcussen, N., Møller, A., Nielsen, K., Nyengaard, J. R., Pakkenberg, B., Sørensen, F. B., Vesterby, A., & West, M. J. (1988) *Acta Pathol. Microbiol. Immunol. Scand.* **96**, 379–394.
47. Yan, Q., Matheson, C., Sun, J., Radeke, M. J., Feinstein, S. C., & Miller, J. A. (1994) *Exp. Neurol.* **127**, 23–36.
48. Anderson, K. D., Alderson, R. F., Altar, C. A., DiStefano, P. S., Corcoran, T. L., Lindsay, R. M., & Wiegand, S. J. (1995) *J. Comp. Neurol.* **357**, 296–317.
49. Nilsson, O. G., Leanza, G., Rosenblad, C., Lappi, D. A., Wiley, R. G., & Björklund, A. (1992) *NeuroReport* **3**, 1005–1008.
50. Torres, E. M., Perry, T. A., Blokland, A., Wilkinson, L. S., Wiley, R. G., Lappi, D. A., & Dunnett, S. B. (1994) *Neuroscience* **63**, 95–122.
51. Berger-Sweeney, G., Hoekers, S., Mesulam, M.-M., Wiley, R. G., Lappi, D. A., & Sharma, M. (1994) *J. Neurosci.* **14**, 4507–4519.
52. Kordower, J. H., Chen, E.-Y., Mufson, E. J., Winn, S. R., & Emerich, D. F. (1996) *Neuroscience*, in press.

Immortal Time: Circadian Clock Properties of Rat Suprachiasmatic Cell Lines

David J. Earnest,* Fong-Qi Liang, Matt Ratcliff, Vincent M. Cassone

Cell lines derived from the rat suprachiasmatic nucleus (SCN) were screened for circadian clock properties distinctive of the SCN *in situ*. Immortalized SCN cells generated robust rhythms in uptake of the metabolic marker 2-deoxyglucose and in their content of neurotrophins. The phase relationship between these rhythms *in vitro* was identical to that exhibited by the SCN *in vivo*. Transplantation of SCN cell lines, but not mesencephalic or fibroblast lines, restored the circadian activity rhythm in arrhythmic, SCN-lesioned rats. Thus, distinctive oscillator, pacemaker, and clock properties of the SCN are not only retained but also maintained in an appropriate circadian phase relationship by immortalized SCN progenitors.

Recent progress has unveiled the identities and distribution of putative molecular components of the mammalian circadian clock. Orthologs of the *Drosophila period* gene, *mPer1*, *mPer2*, and *mPer3*, and the mouse *Clock* gene express transcripts that are localized and regulated by light within the known site of the circadian pacemaker in the hypothalamic SCN (1–5). However, the distribution and circadian expression of transcripts encoded by these genes are not restricted to the SCN or to known components of the vertebrate clock (2, 6, 7). The widespread spatial pattern of molecular oscillations leads to at least two critical questions: Is the oscillatory behavior of clock-related genes in peripheral, nonclock tissues strictly indicative of their function as components of the circadian

pacemaker mechanism, and if rhythmicity in the periphery persists independent of SCN regulation, then what is the function of these molecular oscillations within the SCN? In essence, what specific properties distinguish an “oscillator,” a “pacemaker,” and a “clock”?

To explore this issue, we generated immortal cell lines from the anlage of the rat SCN. Similar to other neural cell lines, immortalized SCN cells are characterized by the conservation of many biochemical properties that distinguish mature parental cell types. For example, these lines express neurotrophins and neuropeptides found within the SCN *in vivo* (8). If immortalized SCN progenitors also retain the distinctive functional properties of the SCN, then indices of their cellular activity should oscillate with a circadian periodicity and recovery of circadian behavioral rhythmicity should occur after their transplantation into arrhythmic, SCN-lesioned animals.

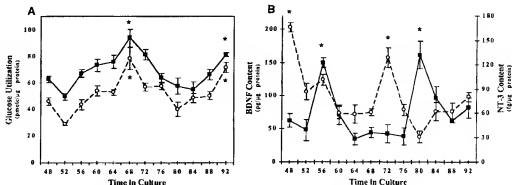
Because rhythmic utilization of 2-deoxyglucose (2-DG) is a well-documented circadian property of SCN activity (9, 10), immortalized

cells were assessed for evidence of oscillatory behavior in this index of cellular metabolism (11). The capacity of immortalized cells to generate circadian rhythms in the expression of brain-derived neurotrophic factor (BDNF) and neurotrophin-3 (NT-3) was also examined concurrently (12) because these neurotrophins are found in the SCN and BDNF levels oscillate on a circadian basis *in vivo* (13, 14). Glucose utilization in immortalized SCN cells was characterized by robust circadian rhythmicity in both the uptake of 2-DG and phosphorylation to 2-DG-6-phosphate (2-DG-6P) for two cycles *in vitro* (Fig. 1A). Throughout both cycles, 2-DG-6P concentrations were consistently maintained at 70 to 80% of the values for 2-DG uptake. The circadian profiles of 2-DG and 2-DG-6P concentrations in immortalized cells were contemporaneous, with peak values at 68 and 92 hours after plating of the cultures. Maximal concentrations of 2-DG and 2-DG-6P were two to three times greater than the corresponding minimum for both rhythms. In contrast to the rhythmic profiles of 2-DG uptake and 2-DG-6P concentrations, accumulation of labeled free 2-DG and glycogen remained at constant basal concentrations in immortalized cells. SCN2.2 cells also exhibited circadian fluctuations in BDNF and NT-3 content, with a three- to sixfold difference between peak and minimum levels of these neurotrophins (Fig. 1B). The circadian patterns of BDNF and NT-3 content were expressed in an inverse phase relationship; when BDNF content reached peak values, NT-3 levels were near their minimum. The circadian maxima in NT-3 content recurred 8 hours in advance of the rhythmic crest in BDNF levels. Despite the differences in their circadian profiles, the rhythms in glucose utilization and neurotrophin expression were invariably locked in the same phase relationship with regard to each other and the time of plating across different experiments. On three separate occasions, the inaugural crests in NT-3 and BDNF levels and glucose utilization occurred at 48, 56, and 68 hours, respectively, after cell plating, with recurrent peaks every 24 hours thereafter. The 12-hour antiphasic relationship between the

D. J. Earnest, F.-Q. Liang, M. Ratcliff, Department of Human Anatomy and Medical Neurobiology, College of Medicine, Texas A&M University Health Science Center, College Station, TX 77843-1114, USA; V. M. Cassone, Department of Biology, Texas A&M University, College Station, TX 77843-3258, USA

*To whom correspondence should be addressed. E-mail: dearnest@tamuc.edu

Fig. 1. Circadian patterns of glucose utilization and neurotrophin expression in confluent cultures of SCN2.2 cells ($N = 5$). (A) 2-DG (solid line, ■) uptake and conversion to 2-DG-6P (dashed line, ○). (B) BDNF (solid line, ■) and NT-3 (dashed line, ○) content. Symbols denote mean (\pm SEM) determinations at 4-hour intervals. Asterisks indicate sampling intervals in (A), during which 2-DG and 2-DG-6P values were significantly greater ($P < 0.05$) than those observed during the three preceding intervals, and in (B), during which peak values for BDNF and NT-3 content were significantly greater ($P < 0.05$) than the three succeeding minima.



rythms of glucose utilization and BDNF content in immortalized SCN cells is similar to that observed in the SCN in vivo; the rat SCN is characterized by maximal 2-DG uptake at circadian time (CT) 6 (9) and peak BDNF content around CT 18 (14). Any potential association between the growth cycle and oscillatory behavior of SCN2.2 cells is unlikely because their generation time of 28 hours is distinctly longer than the circadian periodicity of the glucose utilization and neurotrophin rhythms. Furthermore, this cell line is distinguished by the arrest of proliferative activity and DNA synthesis upon establishment of contact between neighboring cells in confluent cultures (8).

The neural transplantation technique was also used to examine circadian pacemaker and

clock properties of SCN2.2 cells in vivo (15). In these experiments, immortalized cells were grafted near the ablation site in SCN-lesioned rats exhibiting arrhythmic or ultradian rhythms in their wheel-running activity for at least 6 weeks ($N = 10$). The circadian rhythm of wheel-running behavior was restored within 4 to 10 days after transplantation in five of the arrhythmic hosts receiving SCN2.2 cell grafts (Fig. 2A). In these animals, the free-running period of the restored rhythms (mean = 24.02 ± 0.02 hours) was typically shorter than that observed before ablation of the SCN (mean = 24.12 ± 0.09 hours). Complete destruction of the host SCN was confirmed by the absence of immunostaining for vasoactive intestinal polypeptide (VIP), gastrin-releasing

peptide (GRP), and arginine vasopressin (AVP) (16). Functional correlates of SCN2.2 cell grafts in promoting the recovery of circadian rhythmicity were correlated with graft viability and the expression of SCN-like phenotype. Animals with restored circadian behavior were distinguished by viable grafts expressing neurotrophins or neurotrophins found in the SCN in vivo (13, 17). These SCN2.2 cell grafts were located in the host third ventricle region as a single aggregate containing small clusters of VIP-, GRP-, AVP-, or BDNF-immunopositive perikarya (Fig. 3). In contrast, immortalized cell grafts that failed to restore circadian rhythmicity in the remaining SCN-lesioned rats ($N = 5$) were characterized by low cell survival or were devoid of SCN-like elements. The restoration of circadian wheel-running behavior was not observed in SCN-lesioned animals that received control transplants of either F1A-immortalized mesencephalic cells ($N = 6$) or NIH 3T3 fibroblasts ($N = 6$; Fig. 2B), although viable grafts were confirmed in subsequent histological analysis.

Like other cell lines (7) and many peripheral insect and mammalian tissues (6, 18), our immortalized cells derived from the SCN generate circadian rhythms in vitro and thus will be valuable in studying the molecular mechanisms for circadian oscillations. However, our immortalized SCN cells are distinguished by their capacity to confer rhythmicity to the organism, similar to the circadian pacemaker function of the SCN in vivo. These results underscore fundamental distinctions between a circadian oscillator and clock. Whereas an "oscillator" is

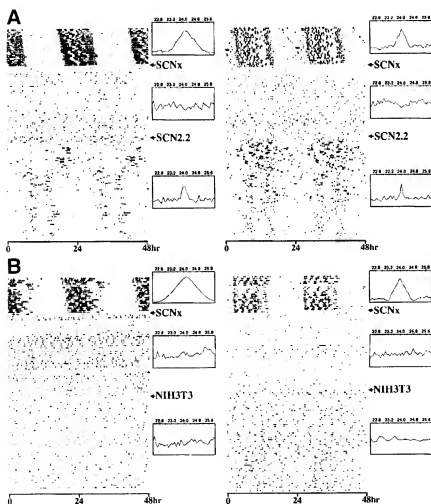


Fig. 2. (A) Restoration of circadian wheel-running activity in two SCN-lesioned rats by grafted SCN2.2 cells. In both intact hosts, the endogenous period of the activity rhythm was 24.1 hours in constant dim illumination. The activity patterns were rendered arrhythmic by bilateral SCN ablation (SCNx), and circadian rhythmicity (period = 24.0 hours) was later restored in both animals by SCN2.2 cell grafts. (B) Activity records of two lesioned, arrhythmic rats receiving grafts of NIH 3T3 cells. Intact hosts exhibited activity rhythms with periods of 24.1 and 24.0 hours. Activity rhythms of both animals were abolished by SCN lesions, and this arrhythmicity persisted after transplantation of NIH 3T3 cells. Periodogram analyses of the data during the last 15 to 25 days of the intact, lesioned, and grafted intervals are shown on the right of each record.



Fig. 3. Photomicrographs depicting immunocytochemical localization of VIP (A) and GRP (B) in transplanted SCN2.2 cells located within the third ventricle of an SCN-lesioned host. Arrows delineate graft location.

merely a device that oscillates, a "clock" represents "a [ajny instrument for measuring or indicating time" and a "pacemaker" is defined as a process or substance that "regulates" the timing of other events (19). Although the capacity to oscillate is a widely distributed property, the restoration of circadian rhythmicity in SCN-lesioned, arrhythmic hosts by immortalized SCN cells but not NIH 3T3 mouse fibroblasts implies that only oscillators derived from the SCN act as pacemakers and have the capability to impose their rhythmicity on mammalian behavior. How these oscillators in the SCN drive rhythms in behavior is unclear at this point, but there is increasing evidence indicating that the SCN secretes a diffusible factor that at least in part contributes to this rhythmic efflux (20). Perhaps one of these factors is a neurotrophin such as BDNF or NT-3, based on their rhythmic expression in immortalized SCN cells and the SCN in vivo (14).

References and Notes

- U. Albrecht, Z. S. Sun, G. Eichele, C. C. Lee, *Cell* **91**, 1055 (1997).
- P. L. Shearman, M. J. Zylka, D. R. Weaver, L. F. Kola-kowski, S. M. Reppert, *Neuron* **19**, 1261 (1997).
- Y. Shigeyoshi et al., *Cell* **91**, 1043 (1997).
- Z. S. Sun et al., *ibid.* **90**, 1003 (1997).
- M. J. Zylka, L. P. Shearman, D. R. Weaver, S. M. Reppert, *Neuron* **20**, 1103 (1998).
- A. Balsalobre, F. Damiola, U. Schibler, *Cell* **93**, 929 (1998).
- D. J. Earnest et al., *J. Neurobiol.*, in press.
- W. J. Schwartz, in *Suprachiasmatic Nucleus: The Mind's Clock*, D. C. Klein, R. Y. Moore, S. M. Reppert, Eds. (Oxford Univ. Press, New York, 1991), pp. 144–156.
- G. C. Newman and F. E. Hospod, *Brain Res.* **381**, 345 (1986).
- The SCN2.2 cell line is derived from fetal progenitors of the rat SCN (embryonic day 15) immortalized with the adenovirus E1A gene (β). Cells derived from a single passage were expanded onto multiple dishes (60 mm) coated with mouse laminin and maintained in minimum essential medium containing 10% fetal bovine serum, glucose (2 mg/ml), and L-glutamine (292 mg/ml) under constant temperature (37°C) and 5% CO₂. At 4-hour intervals for 2 days, confluent cultures ($N = 5$) were incubated for 1 hour with ¹⁴C-labeled 2-DC (0.2 mCi/ml; American Radiological Company, St. Louis, MO). Fractional products of 2-DC metabolism were measured with the methods described by Newman and colleagues [C. C. Newman, F. E. Hospod, C. S. Paliak, *J. Cereb. Blood Flow Metab.* **10**, 510 (1990)]. Fractions derived from 2-DC, 2-DC-6P, free 2-DC, and 2-DC-glycine compartments were placed in scintillation vials in triplicate, dried before addition of scintillant, and then counted on a Beckman scintillation counter.
- Portions (25 μ l) of recovered protein from these samples were also assayed in triplicate for BDNF and NT-3 content by enzyme-linked immunosorbent assay (2). The intra-assay and interassay coefficients of variation were less than 5 and 10%, respectively. BDNF and NT-3 levels were quantified within the linear range of their standard curves (1 to 250 ng/ml and 4.7 to 400 ng/ml, respectively). The antibodies to BDNF and NT-3 in these assays show less than 2% cross reactivity with structurally similar neurotrophins (at 10 ng/ml). Determinations of ¹⁴C-labeled 2-DC uptake and neurotrophin content were normalized for sample protein content as measured by the bicinchoninic acid method (Pierce). This analysis was replicated on three separate sets of cultures. Time-dependent alterations in glucose utilization and neurotrophin levels were identified with one-way analysis of variance, and differences between determinations at distinct time points were tested post hoc for significance with the Newman-Keuls sequential range test.
- F.-Q. Liang, F. Sahraji, R. Miranda, B. Earnest, D. Earnest, *Exp. Neurol.* **151**, 184 (1998).
- F.-Q. Liang, R. Walline, D. J. Earnest, *Neurosci. Lett.* **242**, 89 (1998).
- Male adult Sprague-Dawley rats (150 to 200 g) were housed in individual cages, and wheel-running activity was continuously recorded with Dataquest IV software (Data Sciences, St. Paul, MN). Animals were exposed to a standard 12-hour light/12-hour dark photoperiod (LD) for 7 to 10 days and then maintained under dim constant light (LL; 5 to 10 lux). After 2 to 4 weeks of baseline recording, animals were anesthetized (Xylazine, 2 mg/kg; Ketamine, 10 mg/kg) and with the use of stereotaxic coordinates, SCN lesions were generated by current injection (4 mA for 15 s) through a Teflon-coated tungsten wire (0.203 mm). Animals exhibiting a loss of circadian rhythmicity in their activity behavior for 6 to 8 weeks received transplants of either SCN2.2 cells, E1A-immortalized mesencephalic progenitors, or NIH 3T3 fibroblasts. Colonies of these cell lines were harvested by enzymatic (0.125% trypsin) disaggregation and immediately transplanted as aggregates of 200,000 cells in Hank's balanced salt solution into the periventricular third ventricle region of anesthetized recipients. Some cultures were preincubated with the carboxyanine dye DiI (12 μ g/ml) to allow selective identification of transplanted cells in host brain sections by fluorescence microscopy. Activity records during SCN intact, postlesion, and posttransplantation inter-
- vals were separately analyzed for evidence of circadian rhythmicity with x² periodogram (Tau) and fast Fourier transform analyses. After behavioral analysis, animals were killed with sodium pentobarbital (3.0 mg/kg) and perfused transcardially with 4% paraformaldehyde in 0.1 M phosphate buffer. Animal care and procedures were performed in compliance with state law, federal statute, and NIH policy.
- Brains were prepared for histological processing as described previously (13). Coronal sections (30 μ m) were separately processed for immunocytochemical analysis with antibodies to CRP, VIP (1:1500 and 1:5000, respectively; Peninsula Laboratories, Belmont, CA), AVP (1:10,000; Amel Labs, New York), or BDNF (1:500; Promega, Madison, WI). No immunostaining for these antigens was observed within the host brain or grafts when the primary antisera were omitted or preincubated with homologous antigen (10⁻⁶ M), except for occasional light staining in the area of gliosis surrounding the lesion site.
- R. Y. Moore, *Proc. Fed. Am. Soc. Exp. Biol.* **42**, 2783 (1983).
- J. D. Plautz, M. Kaneko, J. C. Hall, S. A. Kay, *Science* **278**, 1632 (1997).
- American Heritage Dictionary of the English Language (Houghton Mifflin, Boston, MA, 1979).
- K. Silver, J. Lesauter, P. A. Trecco, M. Lehman, *Nature* **382**, 810 (1998).
- We thank D. Bell-Pedersen for comments on the manuscript. This study was supported by NSF grant IBN-9511238 (D.J.E.) and NIH grant NS35822 (V.H.C.).

21 September 1998; accepted 22 December 1998

Horizontal Propagation of Visual Activity in the Synaptic Integration Field of Area 17 Neurons

Vincent Bringuier,* Frédéric Chavane, Larry Glaeser, Yves Frégnac†

The receptive field of a visual neuron is classically defined as the region of space (or retina) where a visual stimulus evokes a change in its firing activity. At the cortical level, a challenging issue concerns the roles of feedforward, local recurrent, intracortical, and cortico-cortical feedback connectivity in receptive field properties. Intracellular recordings in cat area 17 showed that the visually evoked synaptic integration field extends over a much larger area than that established on the basis of spike activity. Synaptic depolarizing responses to stimuli flashed at increasing distances from the center of the receptive field decreased in strength, whereas their onset latency increased. These findings suggest that subthreshold responses in the unresponsive region surrounding the classical discharge field result from the integration of visual activation waves spread by slowly conducting horizontal axons within primary visual cortex.

The average size of the minimal discharge field (MDF) in area 17 neurons is ~2° of visual angle (for the representation near the

area centralis) when it is mapped with a small spot or slit of light (1, 2). The strength of the spiking response results from the amplification of the feedforward thalamo-cortical drive by a local recurrent intracortical loop that preserves the retinotopic mapping of visual input onto cortex (1, 3). However, firing responses to stimuli presented within the MDF can also be modulated by the concomitant stimulation of its surround, over a region up to 10° of relative eccentricity (4, 5). These

Équipe Cognisciences, Institut Alfred Fessard, CNRS, Avenue de la Terrasse, 91198 Gif-sur-Yvette, France.

*Present address: Neurobiology Division, Medical Research Council LMB, 2 Hills Road, Cambridge CB22QH, UK.

†To whom correspondence should be addressed. E-mail: yves.fregnac@iaf.cnrs-gif.fr